

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 0 364 778 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
13.03.1996 Bulletin 1996/11

(51) Int Cl.⁶: **C12P 21/08, C12N 15/06,
C12N 5/20, C07K 1/00,
G01N 33/577, G01N 33/68,
A61K 39/395**

(21) Application number: **89117914.5**

(22) Date of filing: **28.09.1989**

(54) **Antibody against interleukin-1beta**

Antikörper gegen Interleukin-1-beta

Anticorps contre l'interleukine-1 bêta

(84) Designated Contracting States:
CH DE FR GB LI NL

(30) Priority: **01.10.1988 JP 248535/88**

(43) Date of publication of application:
25.04.1990 Bulletin 1990/17

(73) Proprietor: **OTSUKA PHARMACEUTICAL CO.,
LTD.
Chiyoda-ku Tokyo-to (JP)**

(72) Inventors:
• **Uetsuki, Setsuyoshi 3-5, Aza-Nakaseteigai
Itano-gun Tokushima-ken (JP)**
• **Sato, Osamu
Tokushima-shi, Tokushima-ken (JP)**
• **Nakayama, Yasuo
Itano-gun Tokushima-ken (JP)**
• **Hirai, Yoshikatsu
Itano-gun Tokushima-ken (JP)**

(74) Representative: **Hansen, Bernd, Dr.rer.nat. et al
Hoffmann, Eitle & Partner
Patentanwälte
Postfach 81 04 20
D-81904 München (DE)**

(56) References cited:
EP-A- 0 245 052 EP-A- 0 267 611

- **The Journal of Immunology, Vol. 138, No. 12,
15 June 1987 (The Williams & Wiehuls Co,
Baltimore, U.S.A.), pp. 4236-4242; J.S. Kenney
et al: "Monoclonal antibodies to human
recombinant interleukin 1 (IL-1)beta:
quantitation of IL 1beta and inhibition of
biological activity"**
- **EMBO Journal, vol. 7, pp. 339-343 (1988)**

Remarks:

The file contains technical information submitted
after the application was filed and not included in
this specification

EP 0 364 778 B1

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description

The present invention relates to an antibody against interleukin-1 β (IL-1 β), and more particularly to a novel monoclonal antibody against human IL-1 β which antibody makes it possible to immunologically purify, deter-

mine or neutralize IL-1 β .
It is known that human IL-1 is produced not only by macrophages and monocytes but also by various other cells and exhibits diversified molecular forms and biological activities. Presently two kinds of human IL-1 are known which differ in isoelectric point, i.e., IL-1 α and IL-1 β . The primary structures of these interleukins are also clarified (Nature, 315, p.641 (1985); J. Exp. Med., 164, p.237 (1986), etc.).

Extensive research has been conducted on applications of IL-1 as a drug, while attention has been directed to the determination of IL-1 especially in clinical samples, for example, for the research on immunodeficiencies and abnormal immune responses and for the clinical diagnosis of such abnormalities.

At present, bioassay is known as a technique for determining IL-1. With this method, IL-1 is determined in terms of the activity thereof in test samples. The method nevertheless is inefficient to practice and low in accuracy and always involves a need to consider the presence of a factor interfering with the measurement. Additionally, the method has the serious drawback of failing to determine IL-1 α and IL-1 β as distinguished from each other since these interleukins exhibit the same activity.

EP-A-O 267 611, discloses the preparation of a monoclonal antibody which is reactive with human IL-1 β . The antibody is prepared by immunising a mammal with the interleukin serving as the immunising antigen, fusing the immune cells with plasmacytoma cells of a mammal to obtain hybridoma cells, cloning the cells, and selecting the clone which produces the desired antibody.

The Journal of Immunology, 1987, Vol. 138, pp 4236-4242 discloses monoclonal antibodies directed to human recombinant IL-1 β that block the biological activity of IL-1 β by binding to epitopes on IL-1 β important for interaction with its specific receptor.

An object of the present invention is to provide a novel antibody against human IL-1 β .

Another object of the present invention is to provide an antibody against human IL-1 β for use in a novel immunoassay method which is adapted to determine human IL-1 β as distinguished from human IL-1 α and to selectively determine only IL-1 β having biological activity.

Another object of the present invention is to provide an antibody against human IL-1 β which is usable for various diseases involving abnormal production of IL-1 β to inhibit (neutralize) the activity of IL-1 β .

Still another object of the present invention is to provide a technique for producing the above antibody.

The present invention provides a monoclonal antibody against human interleukin-1 β characterized in that the antibody recognizes the conformation formed from

the N-terminus and C-terminus of human interleukin-1 β , and wherein the binding ability of the monoclonal antibody to human interleukin-1 β is correlated to the biological activity of the human interleukin-1 β .

The use of the antibody of the present invention provides a novel method of immunoassay adapted to determine with high sensitivity, high accuracy and ease human IL-1 β having biological activity as distinguished from IL-1 α .

Since the antibody of the invention is specific to human IL-1 β , the use of the antibody provides means for purifying human IL-1 β specifically, for example, by affinity chromatography.

The antibodies of the present invention include an antibody of the type having activity to neutralize the biological activity of human IL-1 β . Whereas abnormal production of IL-1 β is involved in diseases such as chronic articular rheumatism, thyroiditis, hepatitis, nephritis and like chronic inflammatory diseases, arterial sclerosis, Kawasaki disease and like angitis, disseminated intravascular coagulation and blood cancer, the antibody of this type is useful for inhibiting or neutralizing the enhanced biological activity of IL-1 β due to the abnormal production thereof. Thus, the antibody provides a drug which is very valuable for treating these diseases.

The antibody of the present invention can be produced utilizing human IL-1 β as an immunogen. Stated more specifically, the present antibody can be produced by preparing hybridoma cells from plasmacytes (immunocytes) of a mammal immunized with the immunogen and plasmacytoma cells of a mammal, preparing from the hybridoma a selected clone which produces the desired antibody recognizing human IL-1 β , and cultivating the clone.

The human IL-1 β serving as the immunogen for use in the above process is not limited specifically. Useful as such is any of a culture supernatant containing human IL-1 β derived *in vitro* by a known method, purified sample thereof, human IL-1 β prepared by gene recombination techniques, and synthetic peptides having a portion of the amino acid sequence thereof.

Although the mammal to be immunized with the immunogen is not limited specifically, it is desirable to select a suitable animal in view of the compatibility of the plasmacyte with the plasmacytoma cell to be fused therewith. Generally, mice and rats are advantageously usable.

The mammal is immunized by a usual method, for example, by intravenously, intracutaneously, subcutaneously or intraperitoneally administering the immunogen thereto, more specifically, by administering the immunogen (along with a usual adjuvant when desired) to the animal several times every 2 to 14 days at a total dose of, for example in the case of a mouse, about 100 to about 500 μ g/mouse. It is desired that the immunocytes to be used be spleen cells removed from the animal about 3 days after the final administration of the immunogen.

Plasmacytoma cells of mammals useful as the other host cells to be fused with the immunocytes are various known ones, such as p3 (p3/x63-Ag8) [Nature, 256, 495-497 (1975)], p3-U1 [Current Topics in Microbiology and Immunology, 81, 1-7 (1978)], NS-1 [Eur. J. Immunol., 6, 511-519 (1976)], MPC-11 [Cell, 8, 405-415 (1976)], SP2/0 [Nature, 276, 269-270 (1978)], FO [J. Immunol. Meth., 35, 1-21 (1980)], x63.6.5.3. [J. Immunol., 123, 1548-1550 (1979)], S194 [J. Exp. Med., 148, 313-323 (1978)], R210 of rats [Nature, 277, 131-133 (1979)] and like myeloma cells.

The fusion reaction between immunocytes and plasmacytoma cells can be conducted by a known method, such as the method of Milstein et al. (Method in Enzymology, Vol. 73, p.3 (1981)). More specifically, the fusion reaction is conducted in a usual medium in the presence of a usual fusion promoting agent such as polyethylene glycol (PEG) or haemagglutination virus of Japan (HVJ). To achieve an improved fusion efficiency, auxiliary agents such as dimethyl sulfoxide can be added to the medium when required. The immunocyte and the plasmacytoma are used in a usual ratio. For example, immunocytes are used generally in about 1 to about 10 times the amount of plasmacytoma cells. Examples of media useful for the fusion reaction are those usually used for proliferating plasmacytoma cells, such as RPMI-1640 medium and MEM medium, and other media which are generally used for cultivating such cells. It is usually desirable to use the medium without the serum supplement, such as fetal calf serum (FCS). For fusion, predetermined quantities of immunocytes and plasmacytoma cells are thoroughly mixed together in the medium, and a solution of PEG, for example, having an average molecular weight of about 1000 to about 6000 is admixed, as preheated to about 37°C, with the medium usually to a concentration of about 30 to about 60 W/V %. Subsequently, a suitable medium is admixed with the suspension from time to time, followed by centrifuging to remove the supernatant. Repetition of this procedure affords the desired hybridoma.

The desired hybridoma obtained is separated off by cultivation in a usual selection medium such as HAT medium (containing hypoxanthine, aminopterin and thymidine). The cultivation with the HAT medium is conducted for a period of time, usually several days to several weeks, sufficient to extinguish the cells (e.g. unhybridized cells) other than the desired hybridoma cells. The hybridoma cells obtained are then subjected to the usual limiting dilution method to search for the clone producing the desired antibody.

The desired antibody-producing clone can be screened by various methods which are generally used for detecting antibodies, such as the ELISA method (Engvall, E., Method. Enzymol., 70, 419-439 (1980)), plaque method, spot method, agglutination method, Ouchterlony method and radioimmunoassay (RIA). The immunogen is usable for this purpose.

The hybridoma which produces the desired mono-

clonal antibody recognizing human IL-1 β can be subcultured in a usual medium and can be preserved in liquid nitrogen for a prolonged period of time.

The desired antibody can be collected from the culture supernatant of the hybridoma in the usual manner, or from the ascites fluid of a mammal compatible with the hybridoma by administering the hybridoma thereto for proliferation. The former method is suitable for preparing the antibody with a high purity, while the latter method is suited to quantity production of the antibody.

The antibody thus obtained can be further purified by a usual method such as salting out, gel filtration or affinity chromatography.

The monoclonal antibody of the invention prepared in this way has specific reactivity to human IL-1 β .

The antibody of the invention which is of the type having activity to neutralize the biological activity of human IL-1 β is well suited to the specific determination of human IL-1 β having biological activity. Further the antibody of the type having such neutralizing activity, especially the antibody of the type which recognizes the site on the IL-1 β molecule participating in the binding to an IL-1 receptor, is suited to application to the aforementioned diseases involving abnormal production of IL-1 β .

The present invention provides a monoclonal antibody specific to human IL-1 β . The use of the antibody of the invention provides a method of immunoassay which is exceedingly high in determination sensitivity and excellent in specificity and which is therefore adapted to accurately determine human IL-1 β having biological activity and present at a very low concentration in samples such as clinical samples.

The present invention will be described in greater detail with reference to the following examples, which nevertheless in no way limit the invention.

Example 1

Preparation of the present antibody and its characterization

Human [Ser71] IL-1 β (Seikagaku, 58, No.8, p.840 (1986); EPO No.187991) prepared by gene recombination techniques was intraperitoneally administered to a BALB/c mouse at a dose of 1 to 10 μ g every day for 4 weeks. Three to four days after the final immunization, cells were fused by the conventional method (see, for example, Method in Enzymology, 73, p.3 (1981)). For cell fusion, the immunized spleen cell and the myeloma cell (NS-1, Eur. J. Immunol., 6, 511-519 (1976)) were used in the ratio of 5:1 using polyethylene glycol (PEG-1500).

The hybridoma cells were separated off with HAT medium. The resulting supernatant was tested by enzyme immunoassay using a 96-well microplate coated with the above human IL-1 β and peroxidase-labeled goat anti-mouse immunoglobulin antibody (product of E. Y. Lab.) to detect cells producing the desired antibody

against human IL-1 β .

Through repeated cloning by the limiting dilution method, a clone producing the desired antibody was obtained.

The clone (hybridoma producing the antibody of the invention) has been deposited with the designation "Anti OCT-43 producing hybridoma cell (GOM43-4)" and deposition number FERM BP-2565 in the Fermentation Research Institute, Agency of Industrial Science and Technology, MITI.

The antibody of the invention (hereinafter referred to as "GOM43-4") obtained from the clone has the following characteristics.

(1) Subclass of the antibody

The subclass of the antibody determined with use of a mouse antibody subclass detecting kit (product of Bio-Rad) was IgG₁ kappa.

(2) Antibody production level

The amount of IgG in the culture supernatant was about 40 μ g/ml when hybridoma cells were grown to the highest density.

(3) EIA titer

The EIA titer determined by the following method was 2000.

Human IL-1 β adjusted to 200 ng/ml was placed into the wells of a 96-well microplate in an amount of 50 μ l/well and allowed to stand overnight at 4°C. The plate was washed with PBS-Tween 20, and a diluted culture supernatant of the hybridoma was placed into the wells (50 μ l/well), followed by reaction at 4°C overnight and washing. Further peroxidase-labeled anti-mouse immunoglobulin (product of Cappel Laboratories, X2000) was placed into the wells in an amount of 50 μ l/well to effect reaction similarly. After washing, the activity of bound enzyme was determined by colorimetry using o-phenylenediamine as a substrate. The reciprocal of the dilution ratio of the culture supernatant with OD₄₉₂=0.5 was taken at the EIA titer.

(4) Molecular weight

The hybridoma was intraperitoneally cultured in a mouse, and the ascites fluid was purified into IgG₁ with IgG purifying kit (MOPS Kit, product of Bio-Rad). The product was 1.7 x 10² kd in molecular weight. (The sum of molecular weights of heavy chains and light chains obtained by SDS-PAGE was taken as the molecular weight of the antibody.)

(5) Cross reactivity

The procedure (3) was repeated using human IL-1 α .

adjusted to 20 μ g/ml in place of human IL-1 β . The resulting optical density was not different from the blank value which was determined similarly without using IL-1. This indicates that the antibody of the invention exhibits no cross reactivity with human IL-1 α .

(6) Neutralizing activity

This activity was determined in terms of activity to inhibit growth of tumor cells (GIF activity, Gann Monograph on Cancer Research, 34, 155 (1988)).

GOM43-4 the protein content of which was calculated by the UV method was diluted with a medium to 100 μ g/ml. The serial 2-fold dilutions were performed on a 96-well microplate using 50 μ l/well of the culture medium. IL-1 β adjusted to 40 units/ml was placed into the wells in an amount of 50 μ l/well. Finally, a suspension of human melanoma A375 cells adjusted to 2 x 10⁴ cells/ml was placed into the wells in an amount of 100 μ l/well (final concentration of IL-1 β : 10 units/ml). The mixture was incubated in an incubator at 37°C in the presence of 5% CO₂ for 4 days, and the anti-GIF activity of GOM43-4 was determined.

It was found that 2.5 μ g of GOM43-4 was needed to neutralize 10 units of GIF activity of IL-1 β to 1 unit.

(8) Determination of binding site

In the same manner as used for preparing human IL-1 β (EPO No.187991), the following fragments of human IL-1 β were prepared.

Fragments

| | | |
|----|---------|--|
| 35 | 1-153: | human IL-1 β polypeptide |
| | 1-150: | polypeptide comprising the amino acids No.1 to No.150 in human IL-1 β |
| | 1-144: | polypeptide comprising the amino acids No.1 to No.144 in human IL-1 β |
| 40 | 1-140: | polypeptide comprising the amino acids No.1 to No.140 in human IL-1 β |
| | 4-153: | polypeptide comprising the amino acids No.4 to No.153 in human IL-1 β |
| | 7-153: | polypeptide comprising the amino acids No.7 to No.153 in human IL-1 β |
| 45 | 17-153: | polypeptide comprising the amino acids No.17 to No.153 in human IL-1 β |

To *E. coli* expressing each of human IL-1 β and the above fragments was added 600 μ l of 50 mM Tris buffer (pH 8.0) containing 25 mM of EDTA and 0.1% of lysozyme. The mixture was shaken and then allowed to stand in ice water for 15 minutes. To the mixture was further added 500 μ l of 150 mM Tris buffer (pH 8.0) containing 0.3% Triton X100 and 190 mM EDTA, followed by shaking and then by centrifugation. The supernatant was diluted to fiftyfold with PBS-0.1% BSA to obtain a sample solution, which was subjected to an inhibition as-

say in the following manner.

The antibody of the invention adjusted to 10 µg/ml was placed into a 96-well microplate in an amount of 100 µl/well and allowed to stand at 4°C overnight. After washing the plate with water, BSA was applied to the plate to prevent nonspecific adsorption, and the plate was washed with water. The sample solution was placed into the wells in an amount of 100 µl/well, followed by reaction at room temperature for 2 hours. IL-1β (30 ng/ml) labeled with biotin was further placed into the wells in an amount of 100 µl/well and allowed to stand at 4°C overnight. The plate was then washed with PBS-Tween 20, and peroxidase-labeled streptavidin (product of Bethesda Research Lab., X1000) was placed into the wells in an amount of 100 µl/well for reaction. After washing, the activity of the bound enzyme was determined by colorimetry using o-phenylenediamine as a substrate.

The result is shown in Figs. 1 and 2, in which the concentration of polypeptide is plotted as abscissa, and the absorbance at 492 nm as ordinate. These diagrams show how the polypeptides inhibit binding of biotin-labeled IL-1β to the antibody of the invention.

Figs. 1 and 2 appear to indicate that the antibody of the invention recognizes the conformation formed from the N and C-terminus of human IL-1β.

Example 2

ELISA system of present antibody

(1) Sensitivity in ELISA system

The present antibody (GOM43-4) adjusted to 10 µg/ml was placed into the wells of 96-well microplate in an amount of 100 µl/well and allowed to stand at 4°C overnight. After washing the plate with water, 1% BSA-5% FCS/PBS was applied to the plate (400 µl/well) for blocking to prevent nonspecific adsorption, followed by washing with water. The sample solution diluted with 0.1% BSA/PBS was placed into the wells in an amount of 100 µl/well and allowed to stand at 4°C overnight, followed by washing with PBS-Tween 20. Rabbit anti-IL-1β antibody (5 µg/ml) was then placed into the wells (100 µl/well) and allowed to stand at room temperature for 2 hours. After washing the plate with PBS-Tween 20, peroxidase-labeled goat anti-rabbit IgG antibody (product of Bio-Rad., X20000) was placed into the wells (100 µl/well) to effect reaction at room temperature for 2 hours. After washing the plate, the activity of bound enzyme was determined by colorimetry using o-phenylenediamine as a substrate.

Based on the determination sensitivity for the sample concentration with an absorbance (OD_{492 nm}) of 0.1, the sensitivity of the present antibody for IL-1β was 80 pg/ml.

The determination sensitivity of the antibody for [Ser⁷¹] IL-1β checked in the same manner as above was comparable to the above result.

(2) Correlation with biological activity

[Ser⁷¹] IL-1β adjusted to 0.2 mg/ml (with 20 mM phosphate buffer, pH 7.0) was heated at 50, 55, 60 or 65°C for 0 to 24 hours. The solutions thus treated were tested for sensitivity in ELISA system by the procedure (1) above and also for GIF activity by the procedure of Example 1, (6) to establish the correlation therebetween.

The result is given in Fig. 3, in which the measurement (mg/ml) in the ELISA system is plotted as abscissa vs. the GIF activity measurement (x 10⁻⁶ U/ml) as ordinate to show the correlation.

The correlation coefficient calculated from the diagram was 0.988.

In the same manner as above, the present antibody was checked for sensitivity in ELISA system as correlated with the biological activity of IL-1β. The result was comparable to the above result.

Claims

1. A monoclonal antibody against human interleukin-1β characterized in that the antibody recognizes the conformation formed from the N-terminus and C-terminus of human interleukin-1β, and wherein the binding ability of the monoclonal antibody to human interleukin-1β is correlated to the biological activity of the human interleukin-1β.
2. The antibody of claim 1 which has activity to neutralize GIF activity.
3. The antibody of claim 1 which is obtained from a hybridoma cell line FERM BP-2565.
4. Use of the antibody according to any one of claims 1 to 3 for the purification or *in vitro* determination of human interleukin-1β having biological activity.
5. Use of the antibody according to any one of claims 1 to 3 for preparing a medicament effective in the neutralization of human interleukin-1β.

Patentansprüche

1. Monoklonaler Antikörper gegen humanes Interleukin-1β, dadurch **gekennzeichnet**, daß der Antikörper die vom N-Terminus und C-Terminus des humanen Interleukin-1β gebildete Konformation erkennt, und worin die Bindungsfähigkeit des monoklonalen Antikörpers an humanes Interleukin-1β mit der biologischen Aktivität des humanen Interleukin-1β korreliert ist.
2. Antikörper nach Anspruch 1, der eine Aktivität zur Neutralisierung von GIF-Aktivität hat.

3. Antikörper nach Anspruch 1, der aus der Hybridomzell-Linie FERM BP-2565 erhalten ist.
4. Verwendung des Antikörpers nach einem der Ansprüche 1 bis 3 zur Reinigung oder in vitro-Bestimmung von humanem Interleukin-1 β mit biologischer Aktivität. 5
5. Verwendung des Antikörpers nach einem der Ansprüche 1 bis 3 zur Herstellung eines Medikaments, das bei der Neutralisierung von humanem Interleukin-1 β wirksam ist. 10

Revendications

15

1. Anticorps monoclonal dirigé contre l'interleukine-1 β humaine, caractérisé en ce que l'anticorps reconnaît la conformation formée à partir de l'extrémité N-terminale et de l'extrémité C-terminale de l'interleukine-1 β humaine et dans lequel la capacité de liaison de l'anticorps monoclonal à l'interleukine-1 β humaine est en corrélation avec l'activité biologique de l'interleukine-1 β humaine. 20
2. Anticorps suivant la revendication 1, qui a l'activité de neutraliser l'activité GIF. 25
3. Anticorps suivant la revendication 1, qui est obtenu à partir d'une lignée cellulaire d'hybridome FERM BP-2565. 30
4. Utilisation de l'anticorps suivant l'une quelconque des revendications 1 à 3 pour la purification ou la détermination in vitro d'interleukine-1 β humaine ayant une activité biologique. 35
5. Utilisation de l'anticorps suivant l'une quelconque des revendications 1 à 3 pour la préparation d'un médicament efficace pour la neutralisation de l'interleukine-1 β humaine. 40

45

50

55

FIG. 1

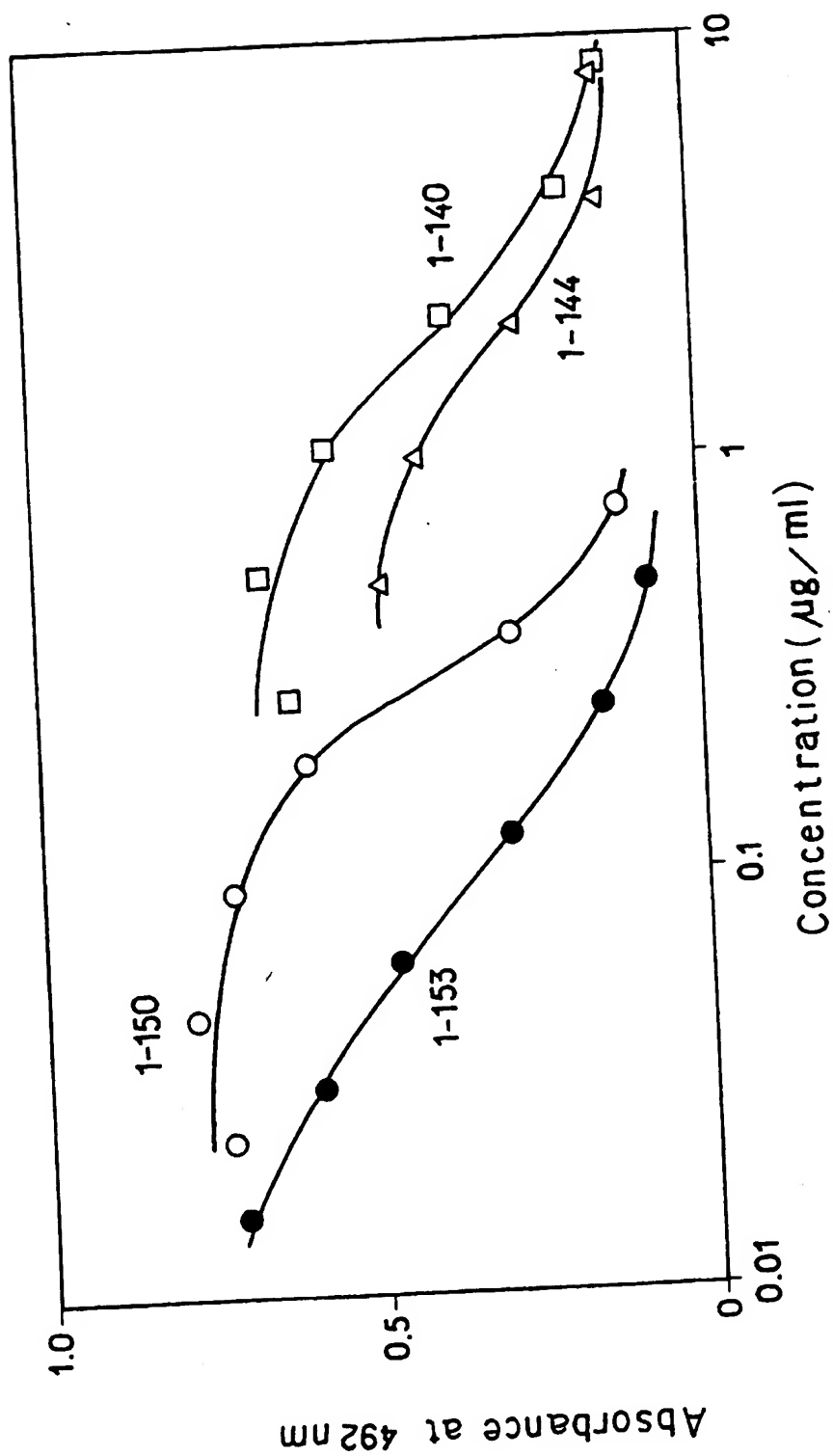


FIG. 2

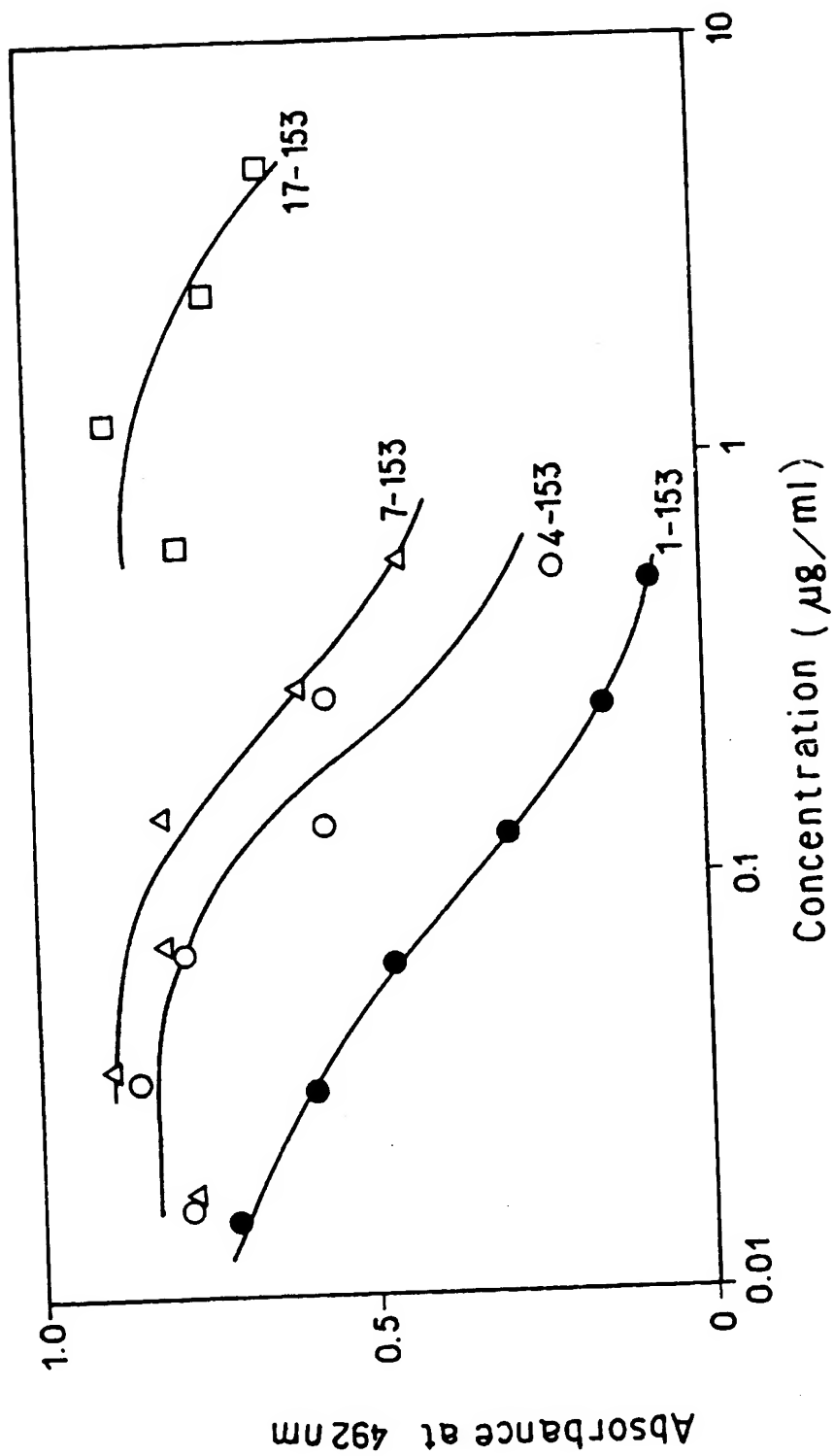


FIG. 3

